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Original article

Astrocyte Alterations in the Hippocampus Following Pilocarpine-induced Seizures in Aged Rats

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ABSTRACT: It is known that the incidence of epilepsy increases with age, but only a few studies have investigated the consequences and mechanisms of seizure and epilepsy in aged animals. Astrocytic changes are known to directly influence neuronal excitability and seizure susceptibility. However, information regarding alterations to astrocytes after seizures in aged animals is lacking in the literature. In the present study, the density and morphology of astrocytes expressing GFAP were investigated in the hippocampus of aged rats that experienced status epilepticus induced by pilocarpine. One month after seizures, astrocytes in aged rats have increased volume and present activated morphology. Despite these morphological changes, the density of astrocytes was not altered in the hippocampus of aged rats after seizures.

Key words: GFAP; Hippocampus; Epilepsy; Aged animals; Gliosis; 3D reconstruction

The prevalence of epilepsy increases after infancy and incidence rates are amongst the highest in the elderly population [1]. Experimental models of seizures and epilepsy in aged animals have yielded variable results regarding seizure susceptibility, neuronal damage and neurophysiological changes [for review see 2]. For example, development of kindling using electrical stimulation of the hippocampus [3] or systemic injection of pentylenetetrazole (PTZ) [4] is delayed in aged rats when compared with adult rats. However, the seizure severity and latency is similar between young adults and aged rats of a strain of genetically epilepsy-prone rats (GEPRs) [5]. On the other hand, aged rats present increased seizure susceptibility to status epilepticus induced by chemical agents, such as kainic acid (KA) [6-9] and pilocarpine [10, 11]. The mechanisms that underlie the discrepancies in the different aging epilepsy

models need to be further elucidated in order to better understand the neuropathological processes that may be unique to the elderly brain.

A growing body of evidence indicates that astrocytes are directly involved with epilepsy development through several mechanisms [12, 13]. It was demonstrated in acute epilepsy models that direct astrocyte stimulation could contribute to neuronal synchronization [14]. It was also demonstrated that astrocytes in the epileptic hippocampus have altered expression of potassium and water channels. Such changes favor an altered water influx and impaired potassium buffering, resulting in facilitation of seizure initiation and development [15, 16]. Glial cells can also contribute to epileptogenesis through the release of inflammatory proteins, predominantly interleukins and chemokines, which can facilitate hyperexcitable conditions [17-19]. Altered

neurogenesis in epileptic animals that may be pro-epileptogenic are also associated with astrocytic changes in the hippocampus [20-23]. A lack of the proper astrocyte association and guidance renders these newborn neurons susceptible to aberrant synaptic targeting, which may contribute to a hyperexcitable condition [24, 25].

During most, if not all neuropathologies, astrocytes exhibit alterations in morphology, number and distribution. Such changes can be investigated by assaying the accumulation of glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed by astrocytes [26]. Considering the scarcity of data on astrocytes in the aging and epileptic hippocampus, the present study investigated astrocytic changes one month after pilocarpine-induced seizures in aged rats.

MATERIAL AND METHODS

All experimental procedures were approved by the IACUC of the University of California, Irvine. Animals were maintained in a 12 hour light-dark cycle with food and water ad libitum.

Seizure induction

Status epilepticus (SE) was induced as previously described [27]. Briefly, aged Sprague-Dawley rats, 22 months old (680-850 gms), were treated with methylscopolamine (1 mg/kg i.p.; Sigma) 30 min before pilocarpine hydrochloride i.p. injection (320 mg/kg; Sigma). Age-matched control rats received saline instead of pilocarpine. Only animals that experienced stage 5 seizures were used for analysis (n=4 per group). It is pertinent to note that in the epileptic group, approximately 50% mortality rate was observed within 2 hrs of pilocarpine treatment. Ninety minutes after SE onset, rats were treated with diazepam (10 mg/kg) to mitigate seizures.

Immunohistochemistry

One month after seizure induction, rats were deeply anesthetized with Euthasol (390 mg pentobarbital sodium and 50 mg phenytoin sodium i.p.) and transcardially perfused with sterile saline followed by 4% paraformaldehyde in phosphate buffer. The brains were removed, postfixed, and sliced in 50 μ m coronal sections using a vibratome. Tissue was processed for immunohistochemistry with mouse anti-GFAP antibody (1:500; Sigma), following the protocol previously described [26].

Astrocyte quantification

The density of GFAP positive astrocytes was estimated using the optical dissector method [28]. Analysis was performed in two different hippocampal regions using a microscope (Nikon Eclipse MU) with a motorized stage connected to a computer running the Stereo Investigator software (MBF Bioscience). The hilus and hippocampal area CA1 were delineated in 5 sections per animal, ranging from bregma -2.16 mm to -4.56 mm. The sections were equally represented within this range to ensure equal representation of the counting areas between the two groups. A counting frame of 40 x 40 μ m was randomly positioned in a lattice of 150 x 150 μ m. Results were statistically analyzed using a Student's t-test and are presented as density of cells / mm³ \pm SEM.

Morphological analysis of astrocytes

For analysis of astrocyte morphology, NeuroLucida software was used (MBF Bioscience). Twenty-five astrocytes in the hilus were randomly selected and traced in their entirety in each experimental group. Cells with clear cell bodies and processes were chosen for reconstruction. The coordinate files generated by the three-dimensional reconstruction were analyzed in the Neuroexplorer component of the NeuroLucida software, generating data of morphological measurements such as total cell surface, volume and process length. The concentric spheres analysis of Sholl [29] was also performed to measure the branching pattern of astrocytes with spheres of 15 μ m.

RESULTS

Astrocyte distribution one month after SE induction

In general, immunohistochemistry for GFAP showed that the principal cell layers, such as the granule cell layer in the dentate gyrus and pyramidal cell layer in CA1 present less astrocytes than the other hippocampal subregions in both control and seizure aged rats (Figure 1 A-D). In addition, astrocytes in the border of the hilus send radial glial-like processes that project from the hilus through the granular cell layer (GCL). However in aged rats with seizures, the radial glial-like processes in the GCL appear to be thinner and less evident than in age-matched controls (Figure 1 A, C). The distribution of the astrocytes in both groups appeared relatively consistent within and between groups, such that individual astrocytes appeared to occupy specified domains with minimal overlap amongst neighboring astrocytes.

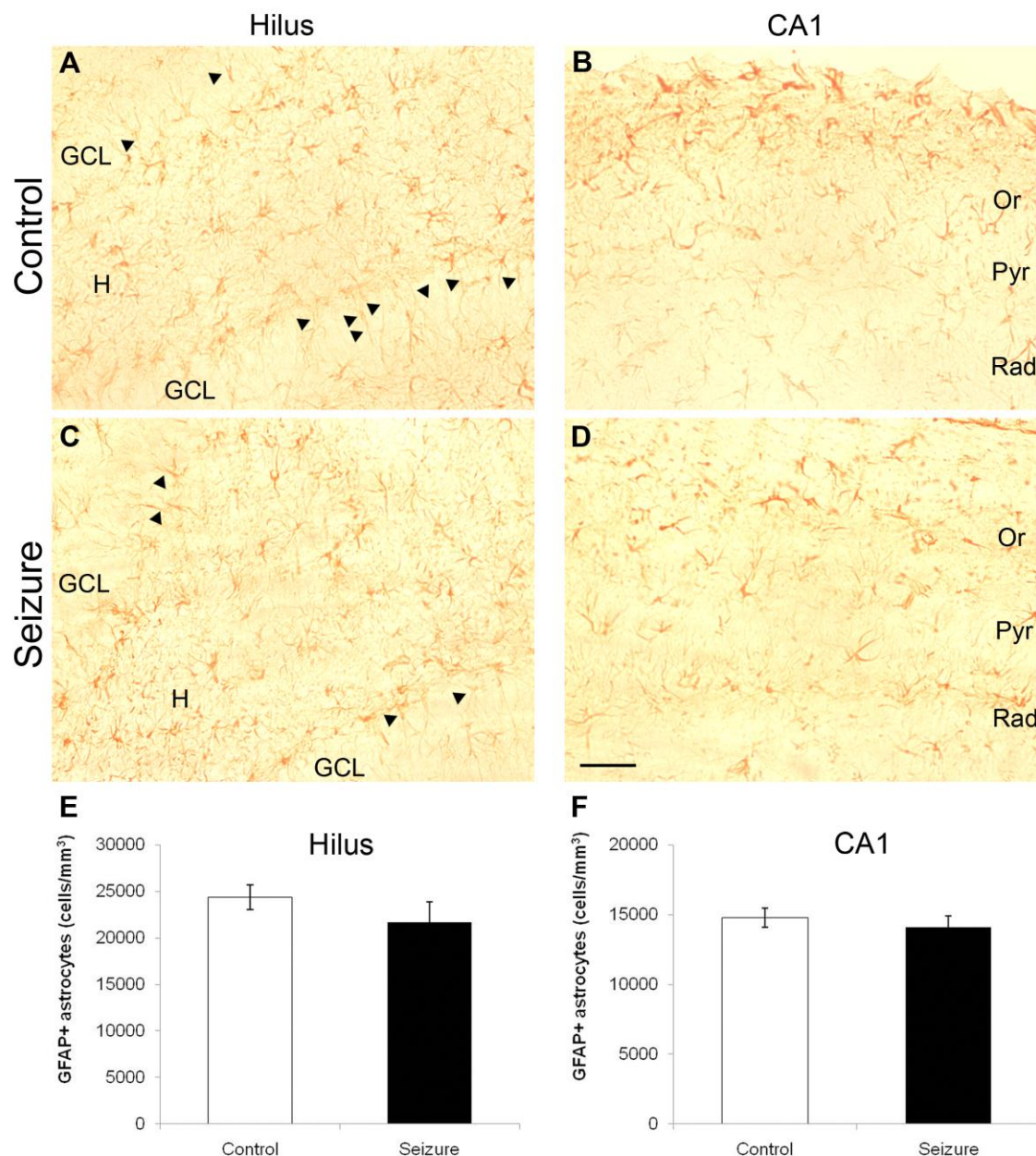


Figure 1. Astrocytes expressing GFAP in the hippocampus. Micrographs showing immunohistochemistry for GFAP in the hilus and CA1 region of control and aged seizure rats (A-D). The distribution and intensity of staining appears very similar between both groups. Less GFAP+ astrocytes are evident in the granule cell layer (GCL) and pyramidal cell layer of CA1 (Pyr), relative to other hippocampal subregions, such as the hilus (H), stratum oriens (Or) and stratum radiatum (Rad), in both control and seizure groups. In addition, astrocytes at the border of the H project radial glial-like processes through the GCL in control rats (arrowheads in A). Note that in aged seizure rats these astrocytic processes in the GCL are thinner and not as evident (arrowheads in C). Graphs depict stereological quantification of astrocytes in the hippocampus (E-F). Consistent with the qualitative analysis, the density of GFAP+ astrocytes in the hilus (E) and CA1 (F) regions was similar between control and aged seizure rats ($p > 0.05$). Scale bar = 100 μ m. Graph values: mean \pm SEM.

Astrocyte density one month after SE induction

Stereological comparison of the density of GFAP+ astrocytes one month after seizures found no significant differences between seizure rats and control rats in either of the two hippocampal regions analyzed (dentate gyrus: $p=0.31$, NS; CA1: $p=0.66$, NS) (Figure 1 E,F). Consistent with the qualitative data, the average distance between neighboring astrocytes in both groups was 35 μm .

Astrocyte morphological analyses

The reconstruction analysis generated data about the morphology of GFAP+ astrocytes (Figure 2 A-D) in the hilus. The results showed no significant differences between the total length of astrocytic processes between groups ($p=0.15$, NS). However, a significant increase in astrocyte cell surface area (Control = $402 \pm 26 \mu\text{m}^2$; Epileptic = $495 \pm 37 \mu\text{m}^2$; $p<0.05$) and cell volume (Control = $83 \pm 6 \mu\text{m}^3$; Epileptic = $118 \pm 11 \mu\text{m}^3$; $p<0.01$) was observed in the hilus (Figure 2), indicating astrocyte hypertrophy in response to pilocarpine-induced seizures. There was no statistical difference in the spherical Sholl analysis (data not shown), indicating a similar spatial distribution of the astrocytic processes in controls and seizure animals.

DISCUSSION

Results from the present study show that one month after pilocarpine-induced seizures, GFAP+ astrocytes exhibit an activated, hypertrophic morphology in aged rats which is not accompanied by increased density of astrocytic cells. These results provide novel data to the literature in aged-epileptic rats and are discussed in the context of a role for astrocytes in the pathogenesis of epilepsy.

Astrocytes play an important role in coupling neuronal organization to blood flow and are actively involved in maintaining, regulating, signaling and altering neuronal synaptic junctions [19, 30]. Moreover, evidence indicates that astrocytes are involved with many neurological dysfunctions, including the pathogenesis of epilepsy.

Overall, the investigation of acute seizures and epilepsy in aged animals is scarce in the literature [2]. Data are also lacking regarding the study of specific astrocytic alterations after seizures in aged animals. However, there is scant evidence using animal models of aging and epilepsy, which are different from the current study, that demonstrate astrocyte activation shortly after seizure induction. For example, one week after systemic KA injection in C57BL/6 mice, hippocampal GFAP levels measured by ELISA were elevated in aged mice.

This was accompanied by increased GFAP immunoreactivity and astrocytic hypertrophy [31]. Similarly, one month after KA injection directly into the mouse hippocampus, the levels of GFAP measured by Western blotting and immunohistochemistry were significantly increased [32]. It is possible that in the pilocarpine-model of epilepsy, a transient change in astrocyte number occurs prior to the 30 day timepoint in aging animals, as has been observed using the pilocarpine model in adult mice and rats [33,34].

Consistent with these findings, it was previously reported that aged rats present an increase in the area of GFAP+ astrocytes 26 days after neurodegeneration and deafferentation induced by subconvulsive dose of intracerebroventricular KA administration [35]. In spite of the astrocyte activation, emergence of nestin positive reactive astrocytes after injury was clearly diminished in the aged hippocampus [35]. In addition, there is a substantial decline of glial derived growth factors, such as IGF-1, VEGF and FGF-2 with aging, possibly a consequence of age-related impairment in synthesis by astrocytes in the hippocampus [36, 37].

While pilocarpine, as shown in the current study, and KA [32, 35] both appear to induce astrocyte activation in aged animals, an absence of astrocytic alterations after seizures in aged rats was shown in a study using PTZ-kindling in senescence-accelerated mice P8 (SAMP8) [38]. Kondziella et al. [38] demonstrated that the progression of PTZ-kindling was similar regardless of the animals' age, but astrocytes of young animals were affected by PTZ-kindling whereas those from aged animals were not. Interestingly, glutamatergic neurons were affected by PTZ-kindling only in older animals [38]. The authors concluded that PTZ-kindling could lead to epileptic seizures without interfering greatly with astrocytic metabolism in aged animals [38]. Another possibility is that glutamatergic neurons might be more susceptible to PTZ-induced seizures in aged animals, perhaps linked to the impaired cytokine production demonstrated in aged rats [36, 37]. Considering the different receptor systems associated with the development of seizures in the different models (e.g. PTZ is GABAergic, kainic acid is glutamatergic and pilocarpine is cholinergic), the discrepancies in the literature may be related to the models chosen and how the affected receptor systems are differentially influenced by aging.

In addition to the synthesis of a milieu of glial derived growth factors, radial-glial like astrocytes in the hippocampal subgranular layer are a major source of newly born neurons in the adult dentate gyrus [39]. In normal adult animals, these radial glial-like astrocytes

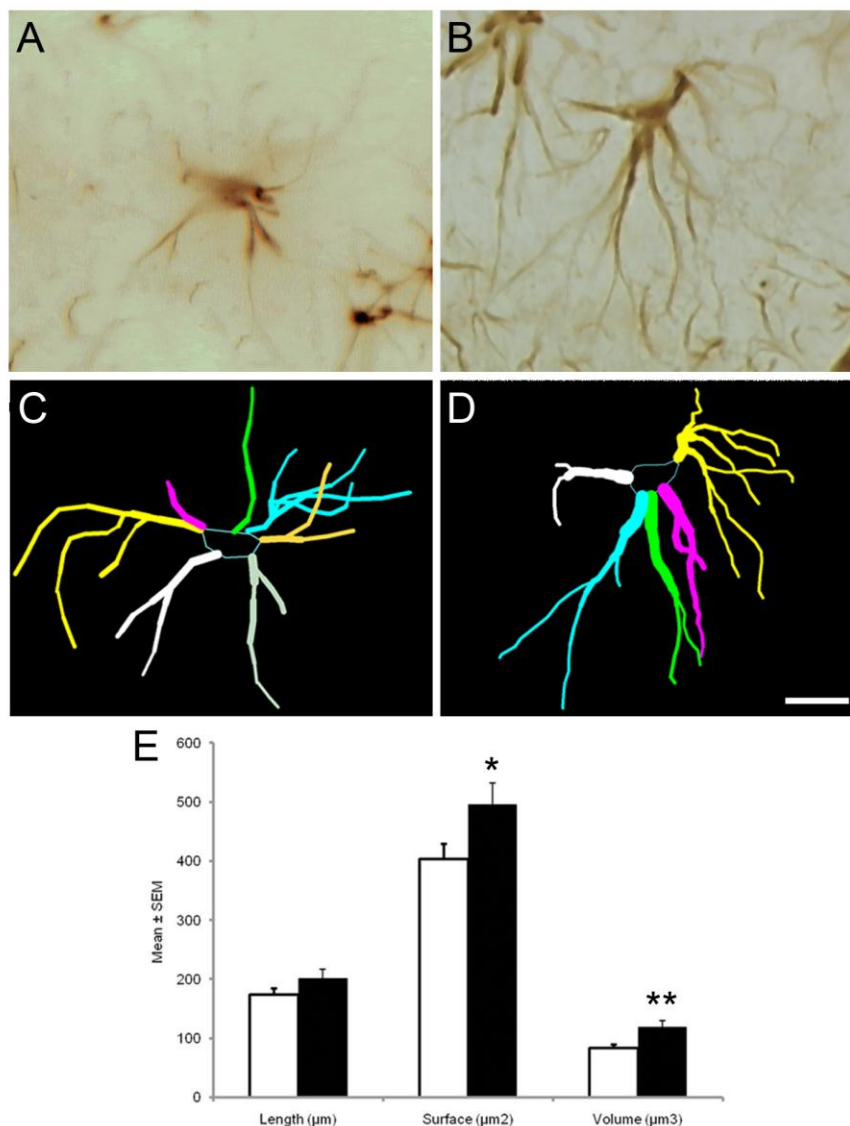


Figure 2. Morphometric analysis of astrocytes in the hilus. An astrocyte from a control (A) animal and its three-dimensional reconstruction (C), compared to an astrocyte from an aged seizure animal (B) and its three-dimensional reconstruction (D). Note that astrocytic processes in the aged seizure animal are hypertrophied when compared with age-matched control. The graph (E) confirms the significant hypertrophy in cell surface and volume in seizure animals (E). White: control animal; black: aged seizure animal. Values: * $p < 0.05$; ** $p < 0.01$. Scale bar: 10 μm .

provide a scaffold for the integration of newborn neurons into the existing granule cell layer [40]. This relationship is altered following pilocarpine-induced seizures in adult rodents where astrocytes modify their morphology and orientation such that an “ectopic glial scaffold” provides an anatomical substrate for hilar basal dendrites to grow into the hilus [21]. In the present study, the radial glial-

like astrocytic processes in the GCL of aged seizure rats appear to be thinner and are less evident than in age-matched controls. Thus, astrocyte activation in the aged hippocampus may be related to the impairment of neurogenesis in the aged dentate gyrus observed after SE (11, 41).

In conclusion, the present data show that one month after pilocarpine-induced seizures in aged rats, there is astrocyte hypertrophy in the hilus but no significant alteration in astrocyte density as revealed by stereological counting of GFAP+ astrocytes.

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Author Disclosure Statement

No competing financial interests exist.

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